DNA Probes, Method and Kit for Identifying Antibiotic-Resistant Strains of Bacteria

This application is a continuation of U.S. Application No. 09/403,609, filed on March 3, 2000, which was a national stage filing under 35 U.S.C. § 371 of International Application No. PCT/DE98/01134 filed on April 22, 1998, the entire contents of each of which are hereby incorporated by reference.

The present invention relates to DNA probes, a method and a kit for identifying antibiotic-resistant strains of bacteria.

The occurrence of antibiotic-resistant strains of bacteria, particularly of streptococcus strains, represents an increasing problem. So far, antibiotic susceptibility tests have been carried out by isolating bacteria and establishing a culture to define the minimum antibiotic inhibitory concentration in a biological test. This method takes at least 1 to 2 days. Well-calculated and thus optimum treatment is not possible within this period. Therefore, there is a need for a faster identification of existing resistances.

The object of the present invention consists in providing products and methods by means of which bacterial strains, particularly streptococcus strains, can be tested fast and reliably for existing antibiotic resistances.

This object is achieved by the subject matters defined in the claims.

The invention is described below by way of penicillin resistance of *Streptococcus pneumoniae*. However, this principle also applies in correspondingly general fashion to bacteria and resistances to other antibiotics. Neisserias and MRSA strains (methicillin-resistant *Staphylococcus aureus*), which do not produce β-lactamase, are mentioned by way of example.

All of the penicillin-resistant *S. pneumoniae* strains have modified penicillin target proteins (penicillin-binding proteins, PBP). The DNA sequences of genes which play a decisive part in the development of penicillin resistance in *Streptococcus pneumoniae* have meanwhile been determined in a number of

penicillin-resistant streptococcus strains. Three genes were identified where differences between sensitive and resistant strains occur in connection with the development of penicillin resistance: PBP2x, PBP1a and PBP2b.

A comparison between the DNA sequences shows within the genes regions which are present in all of the sensitive *S. penumoniae* strains but are modified in resistant strains. In this connection, reference is made to figure 1 which shows that the resistant strains differ more or less markedly from the sensitive strain R6 in the PBP2x gene but also differ among themselves.

Because of the above finding that differences between penicillin-sensitive and penicillin-resistant strains occur within certain genes, the applicant developed DNA probes by means of which resistant and sensitive strains can be differentiated. In this connection, reference is made to figure 4. The probes which are specific to sensitive sequences discriminate genes which code for low-affinity PBP variants responsible for penicillin resistance. The probes which are specific to resistant sequences react with a very frequently occurring class of PBP variants and can also be used for epidemiological purposes.

The applicant identified the following DNA probes:

a) Sensitivity-specific probes for PBP2x (SEQ ID NOS.: 1-8). The numerals in the column "nucleotide" refer to the nucleotides of the published sequence (Laible et al., Mol. Microbial. 5, pp. 1993-2002 (1991)). The numerals in parentheses refer to the codon and the position (1, 2 or 3) within the codon of the structural gene. The number of bases in the nucleotide is given by "meric".

Nucleotide (codon)	oligonucleotide	-meric
314-330 (105.2-110.3)	AGT CAG CAA CGG GTA AG (1)	17
758-774 (253.2-258.3)	AAC GAA CGA TGG ACG GT (2)	17
792-809 (264.3-270.2)	CAT TTC CAG NCC CCT CCA (3)	18 (N:preferably C)
1098-1114 (366.3-372.1)	TGC AGA TGC CAC GAT TC (4)	17
1302-1317 (434.2-439.3)	CTG GTC AGC TTC CTG CG (5)	17
1677-1696 (559.3-566.1)	TGG TTA TCT AGT CGG GTT AA (6)	20
1715-1731 (572.2-577.3)	CTG TAT CGA TGA GTC CG (7)	17
2011-2029 (671.1-677.1)	AAC AGT TCT GCT GAA GAA G (8)	19

b) resistance-specific probes for PBP2x (SEQ ID NOS.: 14-17) (as above; sequences in parentheses are in accordance with the corresponding sections of sensitive strains(SEQ ID NOS.: 20-23))

1065-1084 (355.3-361-3)	(AGG AGA AGT CTT TAA TAG T) TGG AGA ATA NTT CAA TAG N (I)	19 (N:preferably C)
1202-1221 (401.2-407.3)	(CCC TCC TTG AGC AAA AGA TG) GTC TAC TTG AAC AAA AAA TG (II)	20
1549-1566 (517.1-522.3)	(TTG GTA GGG ACG GAT CCG) TTA GTT GGG ACG GAC CCT (III)	18
1759-1776 (587.1-592.3)	(GTG ACG GTC CAA CAA CCT) GTA ACN NTT CAA CAG CCT (IV)	18

c) Sensitivity-specific probes for PBP1a (SEQ ID NOS.: 9-12) (values refer to the nucleotides of the published sequence of the structural gene; Martin et al., EMBO J. 11, pp. 3831-3836 (1992))

(1034-1051)	TAG GAG CAC GCC ATC AGT (specific in most known sequences)	18
1631-1648	GAC GAA ATG CCT ATC TTG	18
1722-1740	CTC TCA ATT TGT AGC ACC T	19
1794-1812	CTA TTC TAA CCG TCT GAC A	19

d) Resistance-specific probes for PBP1a (SEQ ID NOS.: 18-19)(SEQ ID NOS.: 24-25 in parentheses)

945-963	(TAC AGA CGA ATA CGT TGC C) CTC CGA NCA ATA CGT CTC T	19 (N:preferably T)
1735-1754	(GCA CCT GAT GAA CTA TTT GC) GCT CCA GAT NAA ATG TTT GT	20 (N:preferably G)

e) Sensitivity-specific probes for PBP2b (SEQ ID NO.: 13)(values refer to the nucleotides of the published sequence of the structural gene; Hakenbeck, R., Matrin, C., Dowsen, C., Grebe, T., J. Bacterial. 176, pp. 5574-5577 (1996))

1329-1348	ATC AAA TAC CTA TAT GGT CC	20

N = any nucleotide

The above probes and those differing therefrom by one or several nucleotides, preferably up to 4 nucleotides, respectively, are perfectly suited to test unknown *Streptococcus pneumoniae* strains f or resistance to penicillin.

For this purpose, bacteria according to the invention are centrifuged off a sample and in the case of *S. pneumoniae* the PBP genes (the resistance determinants) are amplified directly via PCR (polymerase chain reaction) as described in the literature (Grebe and Hakenbeck (1996), Antimicrob. Agents Chemother. 40, pp. 829-834). The advantage in connection with *S. pneumoniae* consists in that a detergence-induced lysis occurs rapidly and thus PCR can be carried out without long-winding DNA preparations. Since this step fails with other streptococci, only pneumococcus DNA is amplified specifically by means of this step. As an alternative, bacterial DNA (chromosomal and/or extrachromosomal) is isolated according to standard methods. This DNA is hybridized with at least one sensitivity-specific probe and with at least one resistance-specific probe under standard conditions with which a person skilled in the art is sufficiently familiar (see e.g. Maniatis et al., Molecular Cloning, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory). The hybridization is preferably carried out under stringent conditions such as 20^{0}C below the melting point of the hybridizing DNA. The oligonucleotides are preferably chosen such that they have similar melting temperatures and thus several of them can be tested in the same hybridization batch under the same conditions (see figure 2) . The oligonucleotides are preferably labeled when offered (P^{32}, S^{35}, biotin/avidin system; dioxygenine (DIG)-labeled; fluorescein-labeled) and hybridized against immobilized DNA. As an alternative, the oligonucleotides are offered on an oligonucleotide microarray in non-labeled fashion and the DNA to be hybridized is obtained via PCR and labeled while amplified.

It can be concluded from the hybridization result whether or not the unknown strain is sensitive to antibiotics. Depending on the resistance gene, at least one sensitivity-specific probe and one resistance-specific probe should be used for the hybridization. However, the DNA of the unknown strain is hybridized advantageously with several sensitivity-specific and resistance-specific probes in succession, since evaluation of resistance by means of only one

combination of sensitivity-specific probes and resistance-specific probes can be inaccurate and rather only serve as a rough estimate. This applies particularly to the case of penicillin resistances in pneumococci and neisserias.

Preferred hybridization conditions depend on the AT content and length of the oligonucleotides. The person skilled in the art can select suitable conditions on the basis of his technical knowledge. Thus, e.g. 10-100 ng/ml labeled oligonucleotide for PBP2x (see above) are used in SSC hybridization solution at a hybridization temperature of 45°-60° C for at least 5 hours, preferably overnight.

The oligonucleotides can also be used as PCR primers to as to develop a PCR test therewith (see figure 3) . This test can dispense with the somewhat more time-consuming hybridization. However, several PCRs must be used per strain. This method is suitable above all for epidemiological purposes.

The circumstance that less probes are known for PBP1a and particularly for PBP2b follows from the fact that smaller gene regions are of significance for resistance in PBP1a and particularly in PBP2b and therefore also only smaller regions have a sequence variation.

The invention also relates to a kit for carrying out the above method. This kit comprises means for isolating DNA from bacteria and for the PCR amplification of specific resistance determinants, respectively, sensitivity-specific DNA probes and resistance-specific DNA probes (lyophilized and as oligonucleotide microarray, respectively), reagents, solutions, buffers and means for hybridization and the subsequent detection of hybridized DNA. The sensitivity-specific DNA probes and resistance-specific DNA probes are preferably the ones listed above.

The advantage of the present invention is that bacteria, particularly pneumococci, can be assessed as to antibiotic resistance within the shortest time, i.e. within few hours. This enables subsequently a well-calculated and efficient treatment of diseased patients.

The invention is further described by means of the figures showing:

Figure 1 shows a comparison of gene sections of the *Streptococcus pneumoniae* PBP2x gene between penicillin-sensitive and strains; codon 85-750; R6: penicillin-sensitive strain; Others: penicillin-resistant strains.

Figure 2 shows the hybridization on an oligonucleotide array. The arrangement of the probes on the array is indicated in the first block of the figure. Numerals (1) to (8) and (I) to (IV), respectively, correspond to the numbering of the above-mentioned probes for PBP2x.

A) Strain R6, a sensitive S. *pneumoniae* laboratory strain and representative of other sensitive strains: all sensitivity-specific oligonucleotides (Nos. 1-8) are identified whereas all of the four resistance-specific oligonucleotides (I-IV) are not identified.

B) Strain 2349 whose PBP2x gene belongs to a frequently and globally occurring class of PBP2x genes of resistant pneumococci. Only one of the sensitivity-specific oligonucleotides is identified, since the modified sequence does not cover the 3' region of the gene. All of the other sensitivity-specific oligonucleotides (Nos. 2-8) do not hybridize. All of the resistance-specific oligonucleotides (I-IV) hybridize.

C) Strain J19, a resistant strain having a PBP2x which only in part has sequences which correspond with that of strain 2349. One of the resistance-specific oligonucleotides (III) does not respond.

D) Strain Pn12, a resistant strain from Papua, whose PBP2x has an unusual sequence. Five of the sensitivity-specific oligonucleotides do not respond, an evidence for the fact that the PBP2x has no continuous sensitive sequence (and thus conveys resistance) However, the resistance-specific oligonucleotides do not respond either, which indicates that an unusual sequence is also present in the "resistant" gene region. Strains like this one are an exception

but can be detected clearly on account of screening, above all when further oligonucleotides are used which are specific to other PBPs.

Figure 3 shows the result of PCR reactions for the amplification of *S. pneumoniae* R6 DNA as an application on an agarose gel. The PCR primers used were the above PBP2x probes marked above by (1) to (7) as forward primers and probe (8) as reverse primer each. PCRs having probes (I) as forward primer as well as (IV) as reverse primer and (II) as forward primer as well as (IV) as reverse primer, respectively, were carried out as a control. M = size marker. It can be clearly identified that on the gel shown only the sensitivity-specific probes result in an amplification whereas none takes place with resistance-specific probes.

Figure 4 (A-HH) shows detection of the probes according to the invention by sequence comparisons

The invention is further described by means of an example.

EXAMPLE: Isolation of S. *pneumoniae* bacterial DNA and subsequent testing for existing resistance to penicillin

Bacteria of the strain *S. pneumoniae* R6 are inoculated in brain-heart infusion (BHI) broth and allowed to grow at 37^{0}C overnight. The cells were centrifuged off and lyzed by resuspension of the sediment in 10 μl of 10 mM Tris/HC1 buffer, pH 7.2, 0.05 % triton-X100. 1 μl of the cell suspension each are used per 20 μl PCR batch (0.2 μl Taq polymerase, 1 pM oligonucleotide primer each, 2 μl 10X PCR buffer, 4-6 mM $MgCl_2$). 25 cycles with 5 seconds of annealing at 96°C, 5 seconds of annealing at 52° C, 10 seconds of extension at 72° C suffice for the PCR reaction.

A) Agarose gel electrophoresis

The following primer combinations are used in the PCR reactions (conditions see above):

Forward primers	reverse primers
probe (1)	probe (8)
probe (2)	probe (8)
probe (3)	probe (8)
probe (4)	probe (8)
probe (5)	probe (8)
probe (6)	probe (8)
probe (7)	probe (8)
probe (I)	probe (IV)
probe (II)	probe (IV)

The designations of the probes correspond to the numerals for PBP2x, indicated above in connection with the sequences.

In each case, 4 µl aliquots of the PCR reactions were applied onto a 1.5 % agarose gel and separated electrophoretically. The result is shown in figure 3. It follows therefrom that R6 is a sensitive strain.

Bl) Dot blot

S. pneumoniae R6 bacterial DNA is amplified with common primers (Grebe and Hakenbeck (1996), Antimicrob. Agents Chemotherap. 40, pp. 829-834) in a PCR reaction (conditions see above). The PCR-amplified DNA is denatured by heating (2 min. at 96°C, then 4°C), 2 µl thereof each are applied per sample onto a nylon membrane. The DNA is fixed by irradiation with long-wave U.V. light onto the membrane, and non-specific binding sites are saturated at 60°C in prehybridization solution (6x SSC, 5x Denhardt solution, 0.1 % SDS, 50 mM Na phoshphate buffer, pH 6.5, 0.1 mg/ml heringsperm DNA) with mild

shaking for 5 hours. The hybridization with the PBP2x sensitivity-specific oligonucleotide probes (1) to (8) and the resistance-specific probes (I) to (IV), respectively, is carried out in hybridization buffer (like prehybridization solution but with 50 ng/ml oligonucleotide probe) at about 50°C overnight. The filter is washed at room temperature with 2x SSC/0.1 % SDS at 55°C for 2 x 5 minutes. The samples are stained using anti-DIG-AP conjugate in accordance with the instructions from the manufacturer (Boehringer Mannheim) . Here, it also turns out that only the sensitivity-specific probes result in a hybridization, which indicates that the *S. pneumoniae* strain R6 is a penicillin-sensitive strain.

B2) Oligonucleotide microarray

The method is carried out as defined above under El) but with the difference that the oligonucleotides are offered as finished array and the DNA to be hybridized must be labeled via PCR by means of DIG or fluorescein-labeled nucleotides. The principle of high-density microarray hybridization is described in "Nature Biotechnology 14, pages 1675-1680, 1996". The result of this experiment is shown in figure 2A.